

**AMENDMENTS TO THE SPECIFICATION**

In the specification at page 2, line 11, please replace the paragraph which starts with “Cell culture supernatants” with the following amended paragraph:

Cell culture supernatants consist of a broad spectrum of compounds. These include supplements of the cell culture medium such as nonionic block copolymers, particularly the ~~Pluronic~~ PLURONIC family of nonionic block copolymers sold by BASF, and silicon oil, and compounds that are secreted from cells or released after cell lysis (e.g., proteins, lipids). The nonionic block copolymer ~~Pluronic~~ PLURONIC F-68 is usually required as a supplement in cell culture media to protect mammalian cells.

In the specification at page 2, line 17, please replace the paragraph which starts with “In a preferred” with the following amended paragraph:

In a preferred embodiment, the present invention is directed to a process to increase the concentration of cell culture supernatant greatly without significant loss of product macromolecule yield or filterability problems. Applicants discovered surprisingly that a cell culture supernatant comprising the product macromolecules and an organic polymer that co-concentrates with the product macromolecules during ultrafiltration, such as a ~~Pluronic~~ PLURONIC nonionic block copolymer, can be greatly concentrated with higher yields than any reported process of which the Applicants are aware by first subjecting the supernatant to an initial ultrafiltration, then adjusting the conductivity of the retentate, such as by diafiltration with water for injection (WFI), diluent or buffer, and then subjecting the solution to a second ultrafiltration.

In the specification at page 3, line 7, please replace the paragraph which starts with “Preferably, the invention” with the following amended paragraph:

Preferably, the invention pertains to the concentration of aqueous solutions of native or recombinant proteins. The starting solution preferably comprises mammalian or insect cell culture supernatant. The invention further pertains preferably to methods for the concentration of cell culture supernatant comprising a product protein and organic polymers of the ~~Pluronic~~

PLURONIC family of block co-polymers, and more preferably comprising Pluronic PLURONIC F-68 nonionic block co-polymer. The invention can also be practiced using cell culture supernatants containing other organic polymers such as polyethyleneglycols or antifoam compounds. The present methods produce solutions of proteins having high concentration factors (i.e. from 20 fold to 100 fold or higher, preferably from 75 fold to 100 fold or higher).

In the specification at page 3, line 16, please replace the paragraph which starts with "The present invention" with the following amended paragraph:

The present invention is further directed to products, compositions, and intermediates. Applicants discovered that during ultrafiltration, co-concentrated organic polymers such as Pluronic PLURONIC F-68 nonionic block copolymer induce precipitation of macromolecules. It was also discovered that such precipitation depends on the ionic strength of the solution. Thus, in accordance with the present invention, there is provided a method of concentration of a solution comprising macromolecules and organic polymer. The method comprises first concentrating the solution to produce a first retentate solution, adjusting the ionic strength of the first retentate solution using a suitable diluting agent such that any precipitation of solution components induced by the organic polymer is substantially prevented or substantially reduced to obtain a second retentate solution, and then concentrating the second retentate solution by at least 50 fold, preferably at least 100 fold, and still more preferably by more than 100 fold compared to the macromolecule concentration of the starting solution and obtaining yields of from 75-100% of the macromolecule, preferably at least 95.0%, more preferably at least 99.0%, and particularly preferably a yield percent of 99.5 or greater of the macromolecule. In a preferred embodiment, the organic polymer comprises a member of the Pluronic-PLURONIC family of nonionic block copolymers, and more preferably comprises the nonionic block copolymer Pluronic PLURONIC F-68. Optional further process steps can be conducted. For example, the macromolecule product of the inventive method can be subjected to freezing, thawing, and post-thaw filtrating of the refined product to increase the purity or to prepare a desirable therapeutic dosage.

In the specification at page 4, line 3, please replace the paragraph which starts with "In a particular" with the following amended paragraph:

In a particular embodiment, the present invention is directed to solving the problem of ~~Pluronic-induced~~ PLURONIC nonionic block copolymer-induced protein precipitation. In a first step, the cell culture supernatant is concentrated, preferably by ultrafiltration, to a concentration factor where product loss is minimal (for example 20 fold relative to the original concentration). Then, preferably using the same equipment, all or a portion of the concentrate obtained in the first step above is diafiltered against water (WFI) or another a suitable buffer to lower the conductivity to a point where ~~Pluronic-induced~~ PLURONIC nonionic block copolymer-induced product precipitation is substantially prevented, i.e. a conductivity of below 6 mS/cm, and more preferably from 0.5 to 5 mS/cm. As used herein, conductivity measurements are conducted at 22° C. unless described otherwise. Finally, the material is further concentrated to achieve high final concentration factors (e.g. 75-100X relative to the original concentration) with little or no product loss and with minimized bulk protein precipitation. All three steps can be performed in the same equipment. In this case, there is generally little or no increase in complexity and no new material or hardware qualification is necessary. The initial filtration step allows minimization of WFI or buffer consumption during the step of diafiltration. Because the additional volume that has to be filtered during the diafiltration step is therefore low (usually the additional volume required during diafiltration is less than 20%, often less than 15%), the overall process time is not significantly prolonged.

In the specification at page 5, line 25, please replace the Brief Description of the Drawings with the following amended Brief Description of the Drawings:

#### BRIEF DESCRIPTION OF THE DRAWINGS

The accompanying drawings, which are incorporated in and constitute a part of the specification, illustrate a presently preferred embodiment of the invention, and, together with the

general description given above and the detailed description of the preferred embodiment given below, serve to explain the principles of the invention.

FIG. 1 shows ~~exemplary Pluronic~~ the general structure of PLURONIC nonionic block copolymers.

FIG. 2: Experimental set-up for ~~Pluronic~~ PLURONIC F-68 nonionic block copolymer spiking experiments. Samples were taken from typical UF-concentration run (at 1X-50X; 50X (End) sample was taken from final UF concentrate drained from the system). ~~Pluronic~~ PLURONIC F-68 nonionic block copolymer solutions were prepared in a standard cell culture medium (at 0-200 g/l). The result is a matrix of 25 solutions, with final concentration factors of 0.5-25 fold compared to supernatant and ~~Pluronic~~ PLURONIC F-68 nonionic block copolymer concentrations between 0.5 and 125 g/l (assuming complete retention of ~~Pluronic~~ PLURONIC F-68 nonionic block copolymer during UF and 1 g/l ~~Pluronic~~ PLURONIC F-68 nonionic block copolymer concentration in the medium).

FIG. 3: (a) SDS gel electrophoresis followed by silver staining; (1)=IL-2SA standard (5 mg/l); (2)=UF concentrated harvest (25X); (3)=Supernatant of 25X after centrifugation; (4) Pellet re-dissolved in 1 ml buffer; (b) ZAP (western blot stained using anti-hIL-2-antibody); (1)=unconcentrated harvest; (2)=UF concentrated harvest (25X); (3)=Supernatant of 25X after centrifugation; (4)=Pellet re-dissolved in 10 ml.

FIG. 4: Viscosity of ~~Pluronic~~ PLURONIC F-68 nonionic block copolymer solutions prepared in cell culture medium and various ultrafiltration concentrates. Dilution of culture medium was performed with WFI to reach 1-1.5 mS/cm conductivity.

FIG. 5: Precipitation during spiking of ~~Pluronic~~ PLURONIC F-68 nonionic block copolymer into ultrafiltered culture harvest of different concentration as measured by increase in absorbance at 580 nm in a standard cuvette in a standard spectrophotometer (compare also FIG. 2). Material for 25X (End)-sample taken from final drained concentrate.

FIG. 6: Remaining total protein after centrifugation for Pluronic PLURONIC F-68 nonionic block copolymer spiking experiments (compare also FIG. 2). Material for 25X (End)-sample taken from final drained concentrate.

FIG. 7: Remaining IL-2SA in solution after centrifugation for Pluronic PLURONIC F-68 nonionic block copolymer spiking experiments (compare also FIG. 2). Material for 25X (End)-sample taken from final drained concentrate.

FIG. 8: Bulk protein yield after Pluronic PLURONIC F-68 nonionic block copolymer-induced precipitation. Shown are curves for various protein concentrations (compare FIG. 2). Material for 25X (End)-sample taken from final drained concentrate.

FIG. 9: IL-2SA yields after Pluronic F-68 nonionic block copolymer-induced precipitation. Shown are curves for various protein concentrations (compare FIG. 2). Material for 25X (End)-sample taken from final drained concentrate.

FIG. 10: (a) Concentration profile for retained solutes at membrane surface (schematic), (b) Inhomogeneous pressure distribution and consequently inhomogeneous permeate flux in crossflow filtration (from Vogel et al., 2002), "TMP" is transmembrane pressure.

FIG. 11: Pluronic PLURONIC nonionic block copolymer-induced precipitation of total protein and IL-2SA and its reduction by lowered conductivity (spiking experiments). Low conductivity=1-1.5 mS/cm.

FIG. 12: Influence of salt addition to diafiltered low conductivity concentrate on precipitation of bulk protein.

FIG. 13: Influence of pH on precipitation. Concentrated phosphoric acid was used for stepwise decrease of pH of UF/DF/UF 75X concentrate.

FIG. 14: Influence of conductivity on precipitation for two different pH (pH 6.0 adjusted by addition of concentrated phosphoric acid). UF/DF/UF 75X concentrate was used in both cases.

FIG. 15: The UF/DF/UF isolation process scheme according to a preferred embodiment of the invention.

FIG. 16: Performance of the UF/DF/UF isolation process scheme of the invention in comparison to conventional UF process.

FIG. 17: Filterability of concentrated IL-2SA bulk generated by the new UF/DF/UF isolation process scheme in comparison to conventional UF/DF process.

FIG. 18: Influence of high ~~Pluronic~~ PLURONIC F-68 nonionic block copolymer concentrations (75 g/l) on binding of IL-2SA to the cation exchange resin SP Sepharose FF (Amersham Pharmacia Biotech).

In the specification at page 8, line 28, please replace the paragraph starting with "The present invention is particularly" and the following nine paragraphs with the following amended paragraphs:

The present invention is particularly useful with solutions that are comprised of an organic polymer such as a member of the ~~Pluronic~~ PLURONIC family of nonionic block copolymers, and more preferably comprised of ~~Pluronic~~ PLURONIC F-68 nonionic block copolymer, which is an essential component of many cell culture media for protein manufacturing processes. Other organic polymers are also believed to co-concentrate with a desired macromolecule during ultrafiltration and lead to macromolecule or protein precipitation. Thus, the inventive methods apply to concentrate solutions comprising other co-concentrating organic polymers, such as polyethylene glycol ("PEG"), antifoam polymers and other polymers. In most instances, the protein precipitation is believed to occur as a result of the reduced

dielectricity constant of the concentrated solution following ultrafiltration, as well as the increased competition for water molecules required for solvation of the co-concentrated organic polymers and macromolecules.

It is believed that the fundamental mechanism of precipitation of macromolecules induced by increased concentrations of an organic polymer such as, e.g., Pluronic PLURONIC F-68 nonionic block copolymer, applies to solutions comprising other proteins and macromolecules beyond those discussed specifically in the examples. Therefore, the methods of the present invention can be employed with many different organic polymers and macromolecules. The solutions of macromolecules used in the inventive methods may be solutions of any macromolecules, preferably large biological macromolecules, and more preferably proteins. In one embodiment, a process according the present invention can be employed with the recombinant protein interleukin 2 selective agonist ("IL-2SA"). Other recombinant protein products that can be processed using techniques of the present invention include recombinant human Factor VIII sold by Bayer Corp. as Kogenate.TM., recombinant infliximab sold by Centocor, Inc. as Remicade.TM., recombinant abciximab sold by Johnson and Johnson as Reopro.TM., agalfidafe beta sold by Genzyme Inc. as Fabrazyme.TM., recombinant antihemophilic factor sold by Wyeth Ayerst as Refacto.TM. and recombinant antihemophilic factor sold by Baxter Inc. as Recombinate.TM. and any and all of their second and third generation versions, as well as many other protein products.

A main function of Pluronic PLURONIC F-68 nonionic block copolymer is to protect the cells from potential damage caused by sparging (see e.g. Murhammer and Goochee, 1988; Murhammer and Goochee, 1990; Jordan et al., 1994), incorporated herein by reference) which is in turn necessary to ensure sufficient oxygen transfer within production scale bioreactors. Without the addition of protective substances like Pluronic PLURONIC F-68 nonionic block copolymer, cells adhere to the gas-liquid interface (Chalmers and Bavarian, 1991). During rupture of the gas bubbles, the cells are subsequently subjected to very high shear stresses. Depending on the bubble diameter, maximum energy dissipation rates of up to  $9.52 \times 10^7 \text{ J} \cdot \text{m}^{-3} \cdot \text{s}^{-1}$  have been reported, with smaller bubbles for more efficient aeration also creating higher shear stress (Boulton-Stone and Blake, 1993; Garcia-Briones et al., 1994). In comparison, energy

dissipation rates in the order of  $10^4$ - $10^7$  J\*m<sup>-3</sup>\*s<sup>-1</sup> are known to cause cell death in well-defined flow fields (e.g. Schurch et al., 1988, Augenstein et al., 1971).

Pluronic PLURONIC F-68 nonionic block copolymer protects cells from being subjected to this high shear stress by preventing cell adhesion to the air-liquid interface (Garcia-Briones and Chalmers, 1992), which appears to be a result of the lowering of the dynamic surface tension by Pluronic PLURONIC F-68 nonionic block copolymer (Michaels et al., 1995b). Moreover, Pluronic PLURONIC F-68 nonionic block copolymer has been shown to directly interact with the cell membrane, resulting in significantly reduced shear sensitivity (Goldblum et al., 1990; Michaels et al., 1991).

The degree of protection through Pluronic PLURONIC F-68 nonionic block copolymer depends on its concentration in the media. In many cases, 1 g/l is considered optimal in the literature (e.g. Mizrahi, 1984, Maiorella et al., 1988). In the case of IL-2SA, the concentration of Pluronic PLURONIC F-68 nonionic block copolymer is also preferably 1 g/l (0.1%).

#### Structure and Nomenclature of Pluronics PLURONIC Nonionic Block Copolymers

Pluronics PLURONIC nonionic block copolymers generally comprise a hydrophobic polyoxypropylene (PPO) core block between hydrophilic polyoxyethylene (PEO) blocks, thus can be described generally as PEO<sub>m</sub>-PPO<sub>n</sub>-PEO<sub>m</sub> triblock molecules (see FIG. 1). The number of PEO blocks varies for different Pluronics PLURONIC nonionic block copolymers from m=2-130, whereas the number of PPO blocks varies from n=15-67. The nomenclature of Pluronics PLURONIC nonionic block copolymers as supplied by BASF Corp. of Parsippany N.J. includes a letter code for the physical form, i.e. either liquid (L), paste (P) or flake (F). The letter is followed by a 2 or 3 digit number. The first digit, or in case of a 3-digit-code, the first two digits, multiplied by 300 indicate the approximate molecular weight of the hydrophobic PPO part. The last digit multiplied by 10 gives the approximate content of hydrophilic PEO in the whole molecule (e.g. "8" stands for 80% PEO).

#### Physical Properties of Pluronic PLURONIC F-68 nonionic block copolymer



Pluronic PLURONIC F-68 nonionic block copolymer is a solid (flake) with 80% PEO content. The average molecular weight of the whole molecule is about 8.4 kD (BASF Corp., NJ).

Due to the high content of hydrophilic PEO (i.e., 80%), the molecule is more soluble than many other Pluronics PLURONIC nonionic block copolymers (>100 g/l in water at 25° C.; manufacturer's information, BASF Corp, NJ).

In contrast to conventional surfactants, the micellization of amphiphilic triblock copolymers is inherently more complex, and no sharp CMC (critical micelle concentration, the conc. at which micelles are formed for a given temperature) or CMT (critical micelle temperature, the temperature at which micelles are formed for a given concentration) is generally observed. Instead, a broad CMC and/or CMT range is found by light scattering and/or spectroscopic techniques, e.g. with fluorescence probes (Alexandridis and Hatton, 1995). This range is generally >100 g/l. Since the formation of micelles is driven by entropy, and the free energy of micellization is mainly a function of the hydrophobic PPO block, Pluronic PLURONIC F-68 nonionic block copolymer with its mainly hydrophilic character does not readily form micelles in water at RT (Alexandridis et al., 1994). Instead, for concentrations of 10 g/l in water, the CMT is around 45-50 °C., whereas for concentrations of 100 g/l, the CMT is around 30-35 °C. Therefore, for RT, the CMC is >100 g/l (Alexandridis et al., 1994).

Pluronic PLURONIC F-68 nonionic block copolymer is known to cause some additional membrane fouling in the ultrafiltration step often employed for initial concentration/isolation. As discussed in Schulz et al. (1997) referenced supra, during ultrafiltration, a secondary membrane is formed, which properties dominate the retention of organic polymers. As a result, Pluronic PLURONIC F-68 nonionic block copolymer is at least partially retained even if high molecular weight cut-off membranes are used, which is only possible for particularly large products (e.g. 100 kD). Pluronic PLURONIC F-68 nonionic block copolymer is therefore very difficult to remove by filtration processes (Schulz et al., 1997).

For example, data from a process for isolating a recombinant protein (IL-2SA) is provided. Detailed investigations have shown that Pluronic PLURONIC F-68 nonionic block copolymer is co-concentrated during ultrafiltration processes and induces significant protein

precipitation at concentration factors above 20-25 fold (for 1 g/l Pluronic PLURONIC F-68 nonionic block copolymer in the media).

In the specification at page 12, line 18, please replace Examples 2 and 3 with the following amended Examples 2 and 3:

#### Example 2

##### Co-Concentration of Pluronic PLURONIC F-68 Nonionic Block Copolymer

The average molecular weight of Pluronic PLURONIC F-68 nonionic block copolymer is 8.4 kD, which is relatively large. Due to the formation of secondary membranes during ultrafiltration processes and the inherently inhomogeneous conditions along the crossflow channel, the selectivity of conventional UF technology does usually not allow significant separation of molecules in the size range of Pluronic PLURONIC nonionic block copolymer. Even for 100 kD UF membranes, as used for the largest protein products like rFVIII or gp220/350, significant retention and co-concentration of polymers like Pluronic PLURONIC F-68 nonionic block copolymer is usually found (see e.g. Schulz et al., 1997). It can be assumed that the retention coefficient R of Pluronic PLURONIC F-68 nonionic block copolymer during the ultrafiltration process with, e.g., a 10 kD NMWCO (nominal molecular weight cut-off) will be close to 1 (or 100%). Since the Pluronic PLURONIC F-68 nonionic block copolymer concentration in the medium required to obtain adequate cell protection during fermentation is 1 g/l (0.1%), 30 fold concentration would therefore lead to up to 30 g/l (or 3%) Pluronic PLURONIC F-68 nonionic block copolymer in the UF concentrate.

To confirm this hypothesis, retentate samples of various concentration factors (1X = culture supernatant, 2X, 4X, 8X, 16X and approx. 50X) were submitted for analysis by Thin Layer Chromatography (TLC). The resulting Pluronic PLURONIC F-68 nonionic block copolymer concentrations were estimated as 1-2 g/l for 1 and 2X, 4 g/l for 4X, 4-10 g/l for 8X, 10 g/l for 16X and 50 g/l for 50X. Considering the inherent inaccuracy of the TLC method, these

results clearly confirm the almost complete co-concentration of Pluronic PLURONIC F-68 nonionic block copolymer.

This co-concentration of Pluronic PLURONIC F-68 nonionic block copolymer has of course the additional effect of increasing the viscosity of the product solution. As can be seen from FIG. 4, concentrates from several UF runs show indeed similar viscosity profiles (as a function of the assumed Pluronic PLURONIC F-68 nonionic block copolymer concentration) as Pluronic PLURONIC F-68 nonionic block copolymer solutions of known concentration prepared in either culture media or diluted culture media.

### Example 3

#### Spiking Experiments with Pluronic PLURONIC F-68 nonionic block copolymer

Since it has been demonstrated that the end concentration of Pluronic PLURONIC F-68 nonionic block copolymer in ultrafiltration retentate is usually very high, spiking experiments with culture supernatant and concentrates of different concentration factors were performed in order to characterize the influence of these higher Pluronic PLURONIC F-68 nonionic block copolymer concentrations on protein solubility. As can be seen from FIG. 5, spiking Pluronic PLURONIC F-68 nonionic block copolymer into samples of pre-concentrated culture supernatant indeed causes strong precipitation. As expected, the Pluronic PLURONIC F-68 nonionic block copolymer induced precipitation appears to be more severe for a higher concentration factor. FIG. 6 shows the remaining total protein in solution as measured by the Bradford assay (after centrifugation) as a function of concentration factor and Pluronic PLURONIC F-68 nonionic block copolymer concentration. These results are consistent with the A580 measurements and confirm that the protein is precipitating out as a function of the added Pluronic PLURONIC F-68 nonionic block copolymer concentration and the overall concentration factor. As can be seen from FIG. 7, this is in principle true also for the example of the IL-2SA product molecule.

This induction of protein precipitation by increased ~~Pluronic~~ PLURONIC F-68 nonionic block copolymer concentrations is believed to be caused by two effects. First, the binding of water molecules to the hydrophilic PEO blocks of the ~~Pluronic~~ PLURONIC F-68 nonionic block copolymer reduces availability of water molecules for the hydration hull of proteins. Second, the increased ~~Pluronic~~ PLURONIC F-68 nonionic block copolymer concentration decreases the dielectric constant of the medium, which enhances Coulomb interactions between protein molecules. The overall result is a reduction of the electrostatic shielding and therefore a decrease of the capacity of the system to fully solvate the proteins molecules.

Protein starts to precipitate at ~~Pluronic~~ PLURONIC F-68 nonionic block copolymer concentrations of around 20-30 g/l (see FIG. 8). This is consistent with the fact that in conventional process, severe precipitation starts to occur around 20-25 fold concentration, since at 1 g/l starting concentration of ~~Pluronic~~ PLURONIC F-68 nonionic block copolymer in the medium and 100% retention, end concentrations will be 20-25 g/l.

For the example of IL-2SA, the product molecule precipitates at somewhat higher ~~Pluronic~~ PLURONIC F-68 nonionic block copolymer concentrations (see FIG. 9), which explains that the conventional process still had relatively reasonable yields in the UF process itself although high losses occurred in the subsequent steps as a result of the precipitation.

In the specification at page 16, line 15, please replace the paragraph which starts with “In any case,” with the following amended paragraph:

In any case, it has been shown that the hydrodynamic approach to reduce  $c_{wall}$  can only slightly reduce overall protein precipitation induced by ~~Pluronic~~ PLURONIC nonionic block copolymer, thus allowing only marginally higher concentration factors to be achieved without additional yield losses. Therefore, there was a need for a fundamentally new solution, and this problem has been successfully addressed by processes and products according to the present invention.

In the specification at page 16, line 22, please replace the heading starting with “The Influence of” and the subsequent paragraph with the following amended heading and paragraph:

The Influence of Conductivity on ~~Pluronic~~ PLURONIC Nonionic Block Copolymer-  
Induced Protein Precipitation

Under the physiological conditions of cell culture medium and harvest, protein solubility is usually good. However, it was speculated that for the concentrated cell culture supernatant, the high ~~Pluronic~~ PLURONIC Nonionic Block Copolymer concentrations will draw more water away from the hydration hulls of proteins, most likely allowing for thermodynamically driven hydrophobic interactions between proteins and eventually resulting in protein precipitation. In effect, this situation might be characterized by "increased competition" for water molecules required to maintain protein solubility. Since ~~Pluronic~~ PLURONIC Nonionic Block Copolymer cannot be effectively separated, it was tested by further spiking experiments if a reduction in salt content by diafiltration against WFI (water for injection) helps to maintain protein solubility. As can be seen from FIG. 11, by reducing conductivity from 11-12 mS/cm (as in harvest) to about 1-1.5 mS/cm, proteins appear to precipitate only at high ~~Pluronic~~ PLURONIC F-68 nonionic block copolymer concentration.

In the specification at page 18, line 7, please replace the paragraph which starts with "A preferred method" with the following amended paragraph:

A preferred method of the invention comprises ultrafiltration, diafiltration, then ultrafiltration ("UF/DF/UF") (compare FIG. 15). In a first step, the cell culture supernatant is concentrated by ultrafiltration to a concentration factor known not to cause product loss, such as 20X. Then, in the same UF equipment, the concentrate is diafiltered against water (WFI) or buffer to lower the conductivity to the point where ~~Pluronic~~ PLURONIC nonionic block copolymer-induced product precipitation is efficiently prevented. Finally, the material is further concentrated to achieve very high final concentration factors (e.g. 75-100X) without product loss and with minimized bulk protein precipitation. The initial concentration is intended to minimize water/buffer consumption and overall process time. By using this step, the required volume for subsequent diafiltration is only approximately 15% of the starting volume of cell culture supernatant. After diafiltration, concentration is resumed to reach very high final concentration

factors (e.g. >75-100 fold). Due to the lowered ionic strength, the proteins remain in solution and filterability remains high.

In the specification at page 19, line 1, please replace Example 8 with the following amended Example 8:

#### Example 8

Influence of High ~~Pluronic~~ PLURONIC Nonionic Block Copolymer Concentrations on Downstream Processing/Cation Exchange Chromatography

A higher concentration factor achieved by the UF-DF-UF process means that the ~~Pluronic~~ PLURONIC F-68 nonionic block copolymer concentration of the material going into the first purification column will be higher as well, e.g. approx. 75 g/l (or 7.5%) for a 75X target value. Therefore, further studies were performed to evaluate if these high ~~Pluronic~~ PLURONIC nonionic block copolymer concentrations might have a negative impact on the initial downstream purification step. Often the first purification step is a cation exchange chromatography. FIG. 18 shows adsorption isotherms for a standard ion exchange resin at 20° C. with and without additional spiked ~~Pluronic~~ PLURONIC F-68 nonionic block copolymer. Since ~~Pluronic~~ PLURONIC F-68 nonionic block copolymer is an essential media component, no completely ~~Pluronic~~ PLURONIC nonionic block copolymer-free concentrate was available. However, the UF-DF-UF-TCF 75 fold concentrate was diluted up to 160 fold for the measurement of the "control" isotherm, yielding very low ~~Pluronic~~ PLURONIC F-68 nonionic block copolymer concentrations, especially for the initial slope of the isotherm. In contrast, dilution with a 75 g/l ~~Pluronic~~ PLURONIC F-68 nonionic block copolymer solution in pre-diluted media yields 75 g/l ~~Pluronic~~ PLURONIC F-68 nonionic block copolymer end concentration for all points of the isotherm. As can be seen from the figure, both isotherms are very similar, indicating no negative effect of the raised ~~Pluronic~~ PLURONIC nonionic block copolymer-levels on adsorption thermodynamics.

~~Pluronic~~ PLURONIC nonionic block copolymer will be co-concentrated in practically all ultrafiltration processes. As a result, it induces protein precipitation, which usually will start at

about 20-25 fold concentration factor. This "universal" protein precipitation problem leads to yield losses and other problems in the isolation/purification process and prevents the achievement of higher concentration factors.

Using IL-2SA as a model example, it has been demonstrated that reducing the ionic strength of the culture supernatant can efficiently prevent or minimize ~~Pluronic~~ PLURONIC nonionic block copolymer-induced protein precipitation up to very high ~~Pluronic~~ PLURONIC nonionic block copolymer concentrations.

The resulting new isolation scheme (UF/DF/UF) offers an efficient and robust solution to the precipitation problem. It allows the achievement of up to 100 fold concentration (i.e. up to 5 fold higher than in the old process), with maximized yield and improved filterability. This in turn dramatically facilitates further downstream operations

Furthermore, it has been shown that the resulting high ~~Pluronic~~ PLURONIC F-68 nonionic block copolymer concentrations (up to 75 g/l and higher) do not have a negative influence on IL-2SA binding during downstream cation exchange.

Therefore, the UF/DF/UF process scheme appeals as a very suitable "platform technology" for the isolation of proteins from cell culture fermentation. Besides its increased performance, it is robust and easy to implement and utilizes the same standard ultrafiltration equipment and cleaning procedures as conventional UF.

Additional advantages, features and modifications will readily occur to those skilled in the art. Therefore, the invention in its broader aspects is not limited to the specific details, and representative devices, shown and described herein. Accordingly, various modifications may be made without departing from the spirit or scope of the general inventive concept as defined herein and equivalents.

In the specification at page 21, line 9, please replace the paragraph which starts with "Jordan, M.," with the following amended paragraph:

Jordan, M., Sucker, H., Einsele, A., Widmer, F., Eppenberger, H. M.: Interactions between animal cells and gas bubbles: the influence of serum and ~~Pluronic~~ PLURONIC F-68 on the physical properties of the bubble surface. Biotechnol. Bioeng. Vol. 43. 1994.

In the specification at page 21, line 16, please replace the paragraph which starts with “Michaels, J. D., Peterson, J.F.” with the following amended paragraph:

Michaels, J. D., Peterson, J. F., McIntire, L. V., Papoutsakis, E. T: Protection mechanism of freely suspended animal cells (CRL 8018) from fluid-mechanical injury. Viscometric and bioreactor studies using serum, Pluronic PLURONIC F-68 and polyethylene glycol. Biotechnol. Bioeng. 38. 169-180. 1991.

In the specification at page 22, line 1, please replace the paragraph which starts with “Murhammer, D. W.” with the following amended paragraph:

Murhammer, D. W., Goochee, C. F.: Scale-up of insect cell cultures: protective effects of Pluronic PLURONIC F-68. Biotechnology. Vol. 6. 1988.

In the specification at page 22, line 12, please replace the paragraph which starts with “Schulz, C.,” with the following amended paragraph:

Schulz, C., Vogel, J. H., Scharfenberg, K.: Influence of Pluronic PLURONIC F-68 on the Ultrafiltration of Cell Culture Supernatants in: Carrondo et al. (eds). Animal Cell Technology, From Vaccines to Genetic Medicine, Kluwer Academic Publishers. 1997.



In the Abstract of the specification, please replace the Abstract with the following amended Abstract:

The invention provides methods for concentrating a macromolecule from a solution comprising the macromolecule and an organic polymer by first subjecting the solution to ultrafiltration to produce a first retentate solution, then adjusting the conductivity of the first retentate solution such that any protein precipitation induced by the organic polymer is essentially prevented to produce a second retentate solution, and then subjecting the second retentate solution to ultrafiltration. In a preferred embodiment, the conductivity is adjusted by diafiltration against water, suitable diluent or buffer.

Preferably, the invention pertains to the concentration of solutions of native or recombinant proteins. The invention further pertains preferably to methods for the concentration of cell culture supernatant comprising a product protein and organic polymers of the ~~Pluronic~~ PLURONIC family of nonionic block co-polymers, and more preferably comprising ~~Pluronic~~ PLURONIC F-68 nonionic block co-polymer.